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Patients with breast cancers that express estrogen receptor (ER) commonly receive antiestrogen therapy. The efficacy of this treatment depends on close regulation of breast growth by estrogen. However, as breast cancers progress, they often become resistant to estrogens, and most patients no longer respond to antiestrogen therapy. New antiestrogen treatment options are needed, and alternative therapies may derive from findings showing that some ER molecules occur in plasma membranes of breast cancer cells and interact with transmembrane HER-2 growth factor receptors. Expression of HER-2 receptors occurs in many breast cancers, and the protein kinase activity of HER-2 may modulate ligand-independent activation of ER. If active cross-communication between ER and HER-2 receptor occurs and leads to promotion of cancer growth, this axis may offer a new target for therapeutic intervention. We are assessing the nature of membrane ER and its role in promoting the growth of breast cancers. From this work, we hope to develop new treatment options to prevent cancer progression in models of human breast cancer. Since overexpression of HER-2 receptor in breast cancer is associated with failure of antiestrogen therapy in the clinic, understanding the biologic basis of associations between membrane ER and HER-2 receptors may help to improve patient management and enhance survival.

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INTRODUCTION

Breast cancer is the most common malignancy in women in North America and is usually a disease of post-menopausal women (1). In the clinic, endocrine therapy is an important intervention in women with breast cancers that express the estrogen receptor (ER). Treatment with tamoxifen and other antiestrogens has enhanced the survival of breast cancer patients, and these agents are now used in breast cancer prevention. The success of endocrine therapy in breast cancer is dependent on the close regulation of breast cell growth by steroid hormone receptors (1,2). However, as this cancer progresses, it usually becomes resistant to estrogens, and most patients no longer respond to therapy with tamoxifen or other antiestrogens. New information on the existence of an alternate estrogen signaling pathway in breast cancer cells may promote the design of novel and more effective antihormone treatments for human breast cancers (3).

Growth factor receptor malfunction also occurs in malignant progression, with members of the HER-1 (EGF) family frequently implicated in human cancer (1-3, 4-8). The HER (erb B) receptor family includes the HER-2 (erb B2) protein, a 185-kD transmembrane tyrosine kinase encoded by HER-2 oncogene (9-11), the HER-3 protein (12) and HER-4 receptor (13,14). Overexpression of HER-2 or related growth factor receptors is estimated to occur in two-thirds of sporadic breast cancers (1), while HER-2 amplification or overexpression is found in 25-30% of breast cancers in women and 41% of breast cancers in men (15-18). Overexpression of HER-2 is a marker of poor prognosis (15-19) and is associated with failure of antiestrogen therapy (3,20-31).

Receptors for estrogen occur in a family of potentially oncogenic receptors. Sequence similarities between the erb A gene product of avian erythroblastosis virus and ER suggest that these two proteins likely evolved from a common gene (32). Erb A genes cannot induce cell transformation alone, but cooperate with the viral erb B oncogenes in cell transformation (33). With this lineage of cooperativity between erb A and erb B genes, it is not surprising to find reports of significant cross-talk and interaction between erb B (HER) pathways and estrogen receptor signaling (3,24,27,34-36).

It is generally held that the biologic activity of estrogen in the breast is mediated through the specific high-affinity ER located in breast cell nuclei (1,37). In the absence of estrogen, ER is considered to associate with proteins that prevent its interaction with the cell transcription apparatus. Upon estrogen binding, the receptor undergoes an activating conformational change that promotes association with target genes, thus permitting regulation of gene transcription [see FIG. 1]. In addition to the latter pathway, however, estrogen can also induce extremely rapid increases in the levels of intracellular second messengers, including calcium (39,40) and cAMP (41,42), as well as activation of MAP kinase (43,44) and phospholipase (45). The timecourse of these events is similar to those elicited by peptides, lending support to the hypothesis that they do not involve the classical genomic action of estrogen. Both estrogens and growth factor ligands act as mitogens to promote cell growth in the breast, and the cellular effects of these agents sometimes overlap. The molecular details of this cross-talk between ER and erb B receptors are now beginning to emerge, and ER itself may be an important point of convergence (3,24,34-36).

Many of the rapid effects of estrogen are now attributed to the action of the hormone at the cell membrane, and these biologic actions appear to be mediated by membrane receptors that bind estrogen. The isolation and structural characterization of these native macromolecules have not yet been accomplished, and the derivation and functions of this receptor (or receptors) are largely unknown. Since activation of this alternate signaling pathway by estrogens may represent a mechanism by which estrogens regulate proliferation, we have investigated the nature and activity of this membrane response pathway in human breast cancer cells. Classical models of estrogen action that characterize this signaling pathway as solely due to the activity of an intracellular ligand-dependent transcription factor are clearly incomplete and must be modified to include estrogen receptors as significant components of other signaling pathways. As urged by others (40), "these data beg a reevaluation of the relative contributions of genomic and nongenomic activities in ER biology, an activity that is likely to support the development of pharmaceutical agents that exert differential activities in the two pathways".

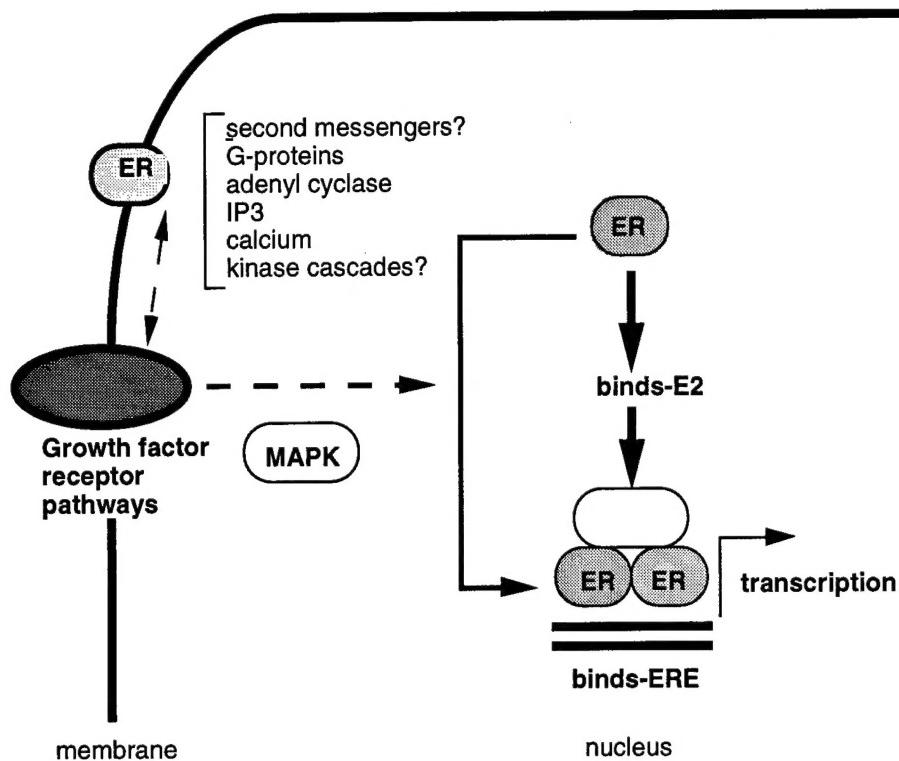


FIG. 1. Postulated cellular mechanism of action of estrogen (E2) and growth factors in breast cancers with estrogen receptor (ER). In most models of estrogen action, estrogen binding to ER in the nucleus promotes receptor dimer formation and receptor phosphorylation that enhances binding to nuclear estrogen-responsive elements (ERE) and coactivator proteins, leading, in turn, to initiation of gene transcription. However, the latter model fails to account for numerous, rapid cell responses to estrogen (41-69). In the hypothesis to be tested here, estrogen may also bind to a membrane ER, with potential for stimulation of estrogenic responses via an alternate pathway. Current reports suggest that membrane-associated ER may activate one or more pathways, including interaction with growth factor membrane receptors such as HER-2 receptor or activation of G-proteins and adenylate cyclase, inositol phosphate, calcium homeostasis and/or MAP kinase. These interactions may promote phosphorylation of ER via estrogen-induced activation of second-messengers and protein kinases or, alternatively, via ligand-independent pathways involving growth factor receptors. Growth of cells treated with estrogen may occur as a consequence of a synergistic feed-forward circuit where estrogen activates cell membrane signaling pathways that act, in turn, to enhance transcriptional activity of ER in the nucleus. Active reconsideration of the classical model of nuclear receptor action is ongoing (38), and the probable importance of alternate signaling pathways is only now beginning to emerge.

RESEARCH PROGRESS

Aim 1) To assess the existence and identity of receptors for estrogen in plasma membranes of human breast cancer cells.

1.a. Enrichment of high-affinity binding-sites with specificity for E₂β in breast cancer cell plasma membranes

To confirm earlier reports of membrane binding-sites for E₂β (52,55,61,63), we measured specific [³H]E₂β binding in subcellular fractions of MCF-7 cells after controlled cell homogenization and fractionation (47,48). With recovery of more than 97% of total E₂β binding found in homogenates of MCF-7 cells, specific [³H]E₂β binding was distributed among crude nuclear, microsomal, mitochondria-lysosome and cytosol fractions (Fig.

2a). After purification of plasma membranes from the crude nuclear fraction by use of discontinuous-sucrose density gradient centrifugation, the PM fraction showed enhanced activity of 5'-nucleotidase, a plasma membrane marker enzyme, to about 23-times that of homogenate (Fig. 2a, b). Specific [³H]E₂ β binding in plasma membranes was enriched to 28-times homogenate activity and represented 22% of homogenate binding. This data shows that specific E₂ β binding co-purifies with a plasma membrane marker protein in membrane fractions from breast cancer cells. LDH activity, highly enriched in cytosol, is not significantly detected in PM (Fig. 2 a, b). In addition, cell DNA recovery was 94 ± 3 % of homogenate levels in nuclear fractions, and no DNA was detected in PM fractions (data not shown).

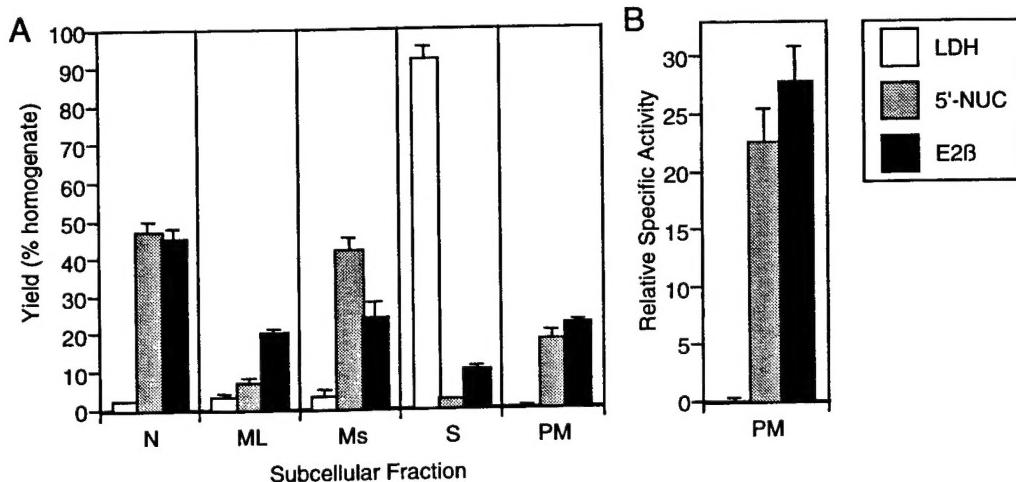


FIG. 2. Distribution and relative specific activities of enzymes and specific [³H]estradiol-17 β binding in plasma membrane and other subfractions of MCF-7 cells. Cells were grown in estrogen-free media prior to harvesting, then disrupted using controlled homogenization methods as before (47,48). A) The yield of marker enzymes and E₂ β binding in each fraction is expressed as % of that in cell homogenate, with mean \pm SE of data from 3 experiments shown. Total recoveries of protein, 5'-nucleotidase (5'-NUC), lactate dehydrogenase (LDH) and specific [³H]E₂ β binding (E₂ β) in crude nuclear (N), mitochondria-lysosome (ML), microsome-rich (Ms) and cytosol (S) fractions ranged from 96-102% of that in homogenates. Homogenate values averaged 34 ± 2 mg/ 10^8 cells for protein; 49 ± 2 nmol/min/mg protein for 5'-nucleotidase; 48 ± 4 units/min/mg protein for LDH; and 240 ± 5 fmol/mg protein for specific [³H]-E₂ β binding. B) Relative specific activity in plasma membrane (PM) represents the specific activity of enzyme or E₂ β binding in a given fraction relative to the homogenate.

Binding of [³H]E₂ β by PM fractions from MCF-7 cells was analyzed further in equilibrium binding studies (Fig. 3). Samples of PM were exposed to [³H]E₂ β concentrations ranging from 1×10^{-10} M to 5×10^{-9} M. As shown in Fig. 2a, all samples with [³H]E₂ β alone retain greater amounts of hormone than paired samples in which [³H]E₂ β was present together with a 100-fold molar excess of unlabeled hormone. The difference between the two curves, representing specific binding of E₂ β , is plotted in Fig. 3b. It is evident that binding of hormone by PM is saturable, and Scatchard analyses of specific [³H]E₂ β binding (cf. 48) show that the dissociation constant for the binding process is 3.6×10^{-10} M. Total binding sites in PM at saturation correspond to approximately 6.7 pmol E₂ β per mg membrane protein. In comparison with the estradiol binding properties of intact MCF-7 cells, plasma membrane estrogen-binding sites retain high affinity for specific estradiol binding and exhibit significant enrichment of ligand-binding capacity (see ref. 3). Further, ligand specificity of [³H]E₂ β binding to PM was established by effective suppression by a 100-fold molar excess of unlabeled E₂ β (Fig. 3b, inset). In contrast, [³H]E₂ β binding by PM was essentially uninfluenced by these levels of estradiol-17 α , progesterone or testosterone.

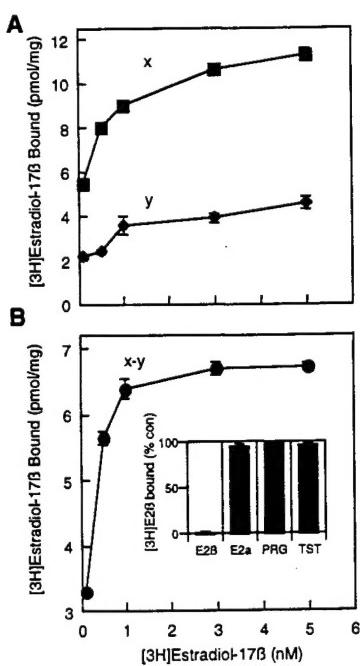


FIG. 3. Binding of [³H]estradiol-17 β by plasma membranes from MCF-7 cancer cells. *A*) Plasma membranes were incubated in Ca⁺⁺-free medium with 0.25 M sucrose with proteinase inhibitors at 50 μ g membrane protein / 2.5 ml for 2 h at 4°C with the concentrations of [³H]E β given alone (curve *x*) or in the presence of a 100-fold molar excess of unlabeled E β plus [³H]E β (curve *y*). *B*) This curve shows the difference between the 2 curves in panel *a* and represents the specific binding of hormone by plasma membranes. In the *inset*, ligand specificity of [³H]estradiol-17 β binding was determined by incubation in the presence of a 100-fold molar excess of competing steroid compounds: E β , E α , progesterone (PRG) or testosterone (TST) as indicated in the graph. Values are shown as mean percent control \pm SE ($n=3$).

1.b. Identification of estrogen receptor forms in subcellular fractions after gel electrophoresis

To characterize putative estrogen receptor forms associated with PM fractions, samples were subjected to Western blot analysis, and blots were probed either with anti-ER antibody Ab2 or with E β -POD (84). PM purified from MCF-7 cells show significant enrichment of a primary 67-kDa protein that reacts strongly with antibody Ab2 to LBD of nuclear ER- α (Fig. 4a).

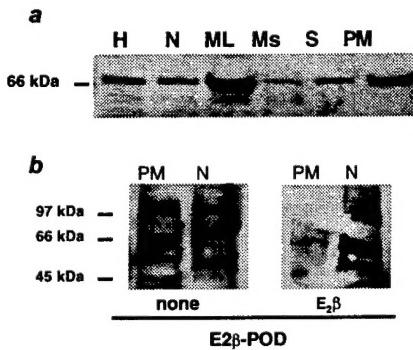


FIG. 4. Identification of estrogen receptor in subcellular fractions of MCF-7 cells by Western blot and ligand blot analyses. Proteins from cell subfractions were analyzed by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. *A*) Immunoblotting with a monoclonal antibody against the LBD of nuclear ER shows the presence of a major 67-kDa band in homogenate (H) as well as in nuclear (N), cytosol (S), mitochondria-lysosome (ML) and microsome (Ms) fractions. A band of similar molecular size shows enrichment in plasma membrane fractions (PM). *B*) Using a ligand blot approach, binding of E β -POD to a 67-kDa band is likewise found enriched in plasma membranes (PM) and in nuclear (N) fractions. E β -POD (estradiol-peroxidase conjugate) binding is shown in the absence (none) and presence (E β) of free estradiol-17 β at a 10-fold molar excess in order to assess specific steroid binding (84).

Similarly, breast cell nuclear fractions are enriched with this protein reactive with ER- α (Fig. 4a). The 67-kDa band also shows evidence of specific labeling with E₂ β -POD (Fig. 4b). A secondary band at 46-kDa and minor bands at 62-kDa and 97-kDa were detected in PM and other cell fractions by use of Western blot (Fig. 4a) and ligand-blotting (Fig. 4b). Using an antibody directed to ER- β , no significant reactivity with proteins at the expected size of 58-62 kDa was found in homogenate, nuclear or plasma membrane fractions of the MCF-7 cells (data not shown).

1.c. Purification of candidate receptors

As indicated above, work aimed at purification of candidate receptors has begun. Further purification will require use of affinity chromatography, with recovered receptor to be used for preparation of monoclonal antibodies and for further molecular characterization and functional studies using cDNA for membrane ER.

Aim 2) To assess the role of membrane estrogen receptors in promoting growth of breast cancers.

2.a. Rapid effects of E₂ β and E₂ β -BSA on activation of MAPK and Akt kinase in breast cancer cells

Post-receptor signal transduction events, such as stimulation of MAPK, extracellular signal-regulated kinase ERK-1 (p44) and ERK-2 (p42) (43,61), may contribute to proliferative effects of E₂ β in breast cells. Thus, we assessed estrogen-induced phosphorylation of MAPK in MCF-7 cells *in vitro*. E₂ β , but not 17 α -estradiol (E₂ α), promotes phosphorylation of MAPK isoforms, with effects evident within 2 min (Fig. 5a).

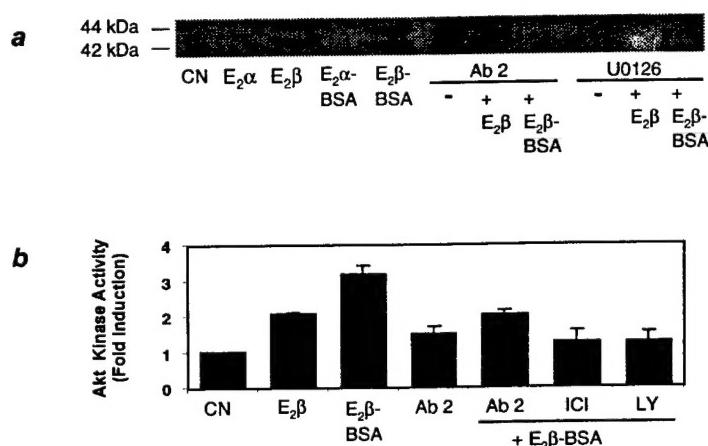


FIG. 5. Post-receptor signal transduction induced by estradiol *in vitro*. A) Treatment of MCF-7 cells with 10 nM estradiol-17 β (E₂ β) induces rapid phosphorylation of mitogen-activated protein kinase (MAPK). E₂ β , but not 17 α -estradiol (E₂ α) or vehicle control (CN), promotes phosphorylation of MAPK isoforms, extracellular signal-regulated kinase ERK-1 (p44) and ERK-2 (p42), with effects evident within 2 min. Similarly, MCF-7 cells treated with E₂ β covalently linked to BSA (E₂ β -BSA, 0.5 μ M), but not to control E₂ α -BSA (0.5 μ M), promoted MAPK phosphorylation within 2 min. Prior treatment with antibody to the LBD of ER (Ab2) blocked the expected response to E₂ β (Ab2 + E₂ β) and to E₂ β -BSA (Ab2 + E₂ β -BSA). In addition, cells were preincubated with U0126, a selective inhibitor of MEK1 and MEK2, before treatment with estrogens, and the inhibitor prevented activation of MAPK by E₂ β (U0126 + E₂ β) and by E₂ β -BSA (U0126 + E₂ β -BSA). B) Akt kinase activation was measured by densitometric analysis of phosphorylated GSK-3 α / β . MCF-7 cells were treated with vehicle (CN) or stimulated with 10 nM estrogen (E₂ β) or 0.5 μ M E₂ β -BSA for 20 min. Cells were preincubated with anti LBD Ab2 (Ab2), ER antagonist ICI 182,780 (ICI) or the PI3-kinase inhibitor LY294002 (LY) before addition of E₂ β -BSA.

To test whether activation of MAPK by E₂β may be mediated by binding of estrogen to membrane-associated receptors, MCF-7 cells were treated with E₂β linked to BSA, a macromolecular complex considered to be membrane-impermeant (52,61). Using E₂β-BSA, but not control E₂α-BSA, phosphorylation of MAPK isoforms is again evident within 2 min of steroid administration. Incubation of cells with antibody against LBD of ER (Ab2) inhibited MAP kinase phosphorylation induced by E₂β or E₂β-BSA. Similarly, we assessed signaling via the phosphatidylinositol-3 kinase (PI3K)/Akt pathway after treatment of MCF-7 cells with E₂β or E₂β-BSA. Both ligands induced significant activation of Akt kinase (Fig. 5b), and inhibition of estrogen-induced effects occurred when cells were preincubated with ER antibody (Ab2), pure antiestrogen (ICI 182,780) or the PI3K inhibitor, LY 294002.

To assess the potential for MCF-7 cell activation by free estradiol liberated from E₂β-BSA, we transfected MCF-7 cells with an ERE-CAT reporter gene as before (3). Cells were exposed *in vitro* to free estradiol-17β or to E₂β-BSA for only 10 minutes, then washed and incubated further. After 24 hours, ERE-CAT reporter gene activity was measured. Short-term treatment with free estradiol-17β stimulated a marked increase in reporter gene activity ($P < 0.001$), but E₂β-BSA elicited no significant effect (Fig. 6).

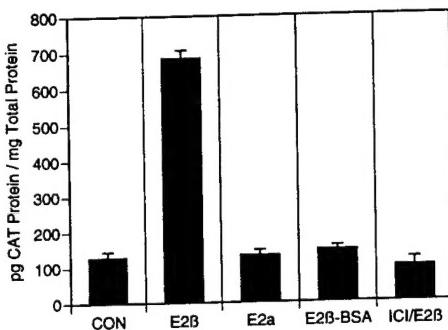


FIG. 6. Activation of an ERE-CAT reporter gene by free estradiol-17β but not by E₂β-BSA. Using established procedures (3), MCF-7 cells were transfected with a reporter plasmid with a palindromic estrogen responsive element (ERE) and the chloramphenicol acetyltransferase (CAT) gene, termed ERE-CAT. Transfected cells were treated with free estradiol (1 nM) or with DCC-treated E₂β-BSA (500 nM) for 10 minutes, washed extensively and incubated further to 24 hours. Thereafter, CAT protein was quantitated in cell extracts and normalized for total protein content in each sample in three independent experiments. In additional control experiments, neither free estradiol nor E₂β-BSA elicited stimulation of a control ERE reporter gene construct transfected in MCF-7 cells as before (3).

Since interaction of E₂β-BSA with plasma membrane binding-sites may be required for intracellular signaling (52,61), we evaluated binding of fluorescein-labeled E₂β-BSA (E₂β-BSA-FITC) in MCF-7 cells. E₂β-BSA-FITC binds at the surface of 77% of MCF-7 cells (Fig. 7a), while only minimal background fluorescence is found among cells incubated with control ligand, BSA-FITC (Fig. 7b). In additional control studies, ER-positive ZR-75 breast cancer cells, as MCF-7 cells, show retention of E₂β-BSA-FITC at the cell surface, but ER-negative MDA-MB-231 breast cancer cells or COS-7 cells do not show significant binding of E₂β-BSA-FITC at the external membrane (not shown). On flow cytometric analysis (Fig. 7e), the E₂β-BSA-FITC complex shows evidence of ligand specificity, with significant reduction ($P < 0.01$) of E₂β-BSA-FITC binding by competition with equi-molar amounts of free E₂β, E₂β-BSA, tamoxifen or ICI 182,780, while the related steroid congener, proges-terone, is not effective. Surface binding of E₂β-BSA-FITC is also significantly diminished by competition with antibody to the LBD of nuclear ER, suggesting some immunologic identity of the membrane site with nuclear ER (Fig. 7c, e). As expected, after permeabilization of cells by disruption of plasma membrane with detergent, intense labeling of ER in cell nuclei is found and occurs in 96% of breast cancer cells (Fig. 7d).

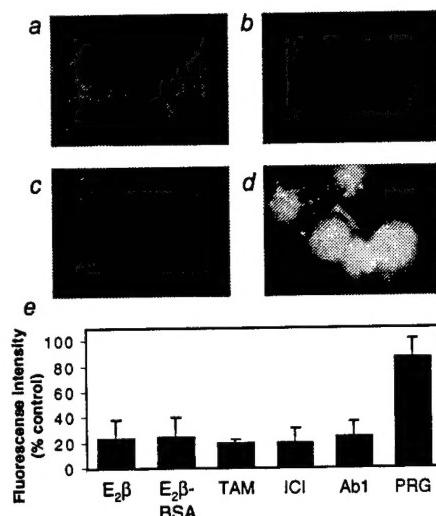


FIG. 7. Estradiol-17 β conjugated to fluorescein-labeled albumin (E₂ β -BSA-FITC) shows binding at the surface membrane of MCF-7 cells. Cells were labeled with 1 μ M E₂ β -BSA-FITC, a membrane-impermeant complex, to assess membrane binding and then analyzed by fluorescent microscopy and flow cytometry. a) Active ligand, E₂ β -BSA-FITC, labels surface membranes of MCF-7 cells. b) Control binding with inactive ligand, BSA-FITC, shows a low level of background cell fluorescense. c) Surface membrane labeling by E₂ β -BSA-FITC is competitively reduced by co-incubation with antibody to LBD of ER (Ab1). d) MCF-7 cells were permeabilized with 0.1 % Triton X-100 to allow visualization of ER binding in the nucleus. e) Flow cytometric analysis of E₂ β -BSA-FITC membrane fluorescense. Cells were incubated with BSA-FITC to assess background fluorescense. With 10,000 cells analyzed per sample, a significant decrease ($P < 0.01$) in fluorescense intensity was observed when cells were incubated with estrogen (E₂ β), E₂ β -BSA, tamoxifen (TAM), ICI 182,780 (ICI) or anti ER antibody (Ab1). No significant competition was observed when cells were incubated with progesterone (PRG). In other control studies, MDA-MB-231 cells with no ER showed no binding or retention of E₂ β -BSA-FITC label, while ZR-75 breast cancer cells with ER expression did show surface binding of the complex (data not shown).

2.b. Inhibition of cell growth in vitro by antibody to ligand-binding domain of ER- α

Since antibodies to cell surface growth factor receptors are sometimes effective in blocking tumor growth (3,77), antiproliferative activity of antibodies to ER- α was evaluated using MCF-7 cells *in vitro*. The estrogen-dependent MCF-7 cells show enhanced growth after treatment with E₂ β , but not E₂ α (Fig. 8a). However, prior exposure to LBD Ab1 or LBD Ab2 elicits a significant reduction ($P < 0.05$) in the E₂ β growth response (Fig. 8a).

Since some recent studies suggest that the proliferative response to E₂ β is committed within 1 min and is evoked by activation of only a small fraction ($\leq 5\%$) of ER (73), we assessed the growth of breast cells after brief treatment with E₂ β -BSA. MCF-7 cells were treated with 0.5 μ M E₂ β -BSA for only 10 min. Then, cells were rinsed and cultivated in estrogen-free media for an additional 72 h. The results show that E₂ β -BSA ($P < 0.001$), but not control E₂ α -BSA, stimulates cell growth (Fig. 8a). Moreover, the proliferative effect of E₂ β -BSA is blocked by treatment of cells with ICI 182,780, a pure antiestrogen ($P < 0.001$) (data not shown), or by prior exposure to anti-ER Ab1 ($P < 0.05$) or Ab2 ($P < 0.001$) (Fig. 8a).

Aim 3) To investigate new treatment options to prevent breast cancer progression in human breast cancer.

3.a. Inhibition of breast tumorigenesis in vivo by antibody to ligand-binding domain of ER- α

The antitumor activity of antibodies to ER- α was evaluated further using MCF-7 tumors *in vivo*. MCF-7 cells were grown as subcutaneous xenografts in female athymic mice primed with E₂ β to promote growth of these estrogen-dependent cells (3). Antibody or control treatments were initiated when tumors grew to > 30

mm^3 . Anti-ER Ab2 was administered in 6 doses over a 26-day period. The results show that antibody to ER, but not control immunoglobulin, elicits a significant suppression of tumorigenesis of human MCF-7 breast cancer xenografts in female nude mice treated concomitantly with E₂ β (Fig. 8b).

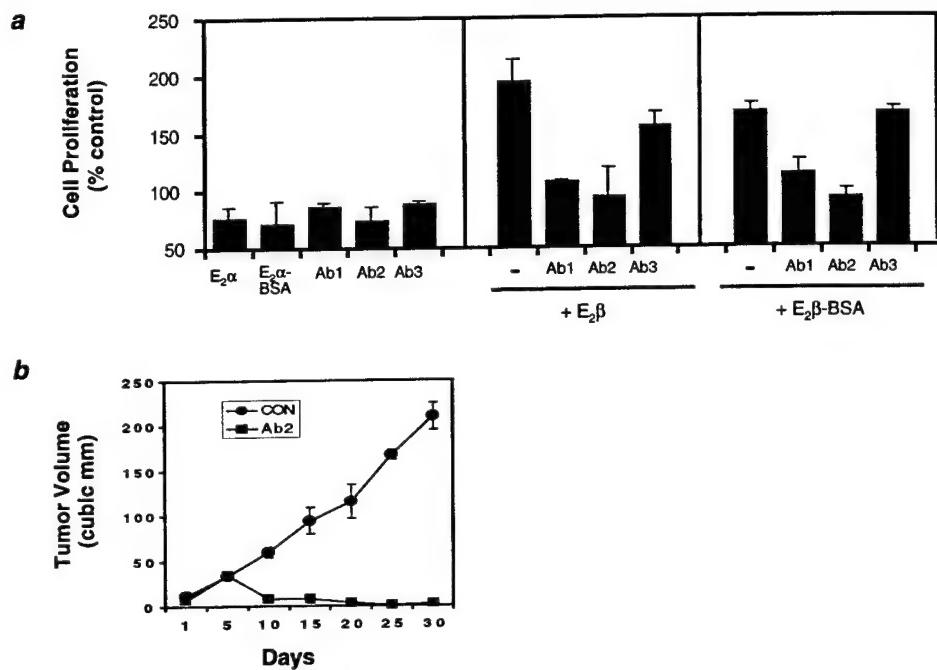


FIG. 8. Inhibition of MCF-7 breast cancer cell growth by a monoclonal antibody directed against the LBD of nuclear ER. *A*) Cells were incubated *in vitro* for 2 h with anti-ER antibodies directed against the LBD (Ab1 and Ab2) or with a control antibody directed to the D and E-domains of ER (Ab3). Thereafter, E₂ β , 17 α -estradiol (E₂ α), E₂ β -BSA or E₂ α -BSA were added to cultures for 10 min. Cells were then cultivated further, and final cell numbers were quantitated after 72 h for each treatment group as indicated. Data (mean \pm SE) were collected from at least 4 independent experiments. *B*) Monoclonal antibody directed against the LBD of ER- α reduces growth of human MCF-7 breast tumor cell xenografts *in vivo*. Female nude mice were primed by treatment with E₂ β subcutaneously, then inoculated with MCF-7 cells as before (3). After 10-14 days, animals with tumors of comparable size were randomized to treatment groups of 6-8 mice. Treatments included IgG isotype-control antibody (CON) or monoclonal antibody directed against the LBD of ER- α (Ab2) administered intraperitoneally twice weekly for a total of 6 doses. After 26 days, no further antibody treatment was given. Tumor volumes were recorded by micrometer measurements, with results shown as mean \pm SE.

KEY RESEARCH ACCOMPLISHMENTS

- Plasma membrane-associated binding sites with high affinity and specificity for estradiol-17 β occur in human breast cancer cells.
- Plasma membrane-associated binding sites for estradiol in human breast cancer cells may play a role in modulating cell growth and survival.

REPORTABLE OUTCOMES

Presentations

1. "Interactions between Type I receptor tyrosine kinases and steroid hormone receptors : Therapeutic implications". Presented at *First International Symposium on Translational Research in Oncology*, Dublin, Ireland (2001).
2. "HER-2 receptor signaling modulates estrogen receptor in breast cancer". Presented at Medical Oncology Seminar Series, UCLA School of Medicine (2001).

Publications

1. Pietras, R.J., Nemere, I. and Szego, C.M. (2001). Steroid hormone receptors in target cell membranes. *Endocrine* **14** : 417-427.
2. Marquez, D.C. and Pietras, R.J. (2001). Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. *Oncogene* (in press).

No abstracts, patents, degrees, development of cell lines, informatics or additional funding or research opportunities to be reported at this time.

CONCLUSIONS

A new approach to cancer therapy involves efforts to cut the lines of communication between hormone receptors and the cell nucleus, thus slowing or blocking cell division. Antiestrogen therapy is one well-known example of this approach, and it is often used to treat breast cancer and to prevent recurrence. Unfortunately, many patients do not respond to current therapy, and almost all treated patients eventually become resistant to antiestrogens. In addition, antiestrogens that are now available can result in abnormal uterine growth and thromboembolic events. The failure of antihormone therapy in the clinic is due to many factors, including the emergence of estrogen-independent growth that is no longer responsive to treatment with antiestrogen agonists.

New options for antiestrogen treatment are clearly needed, and alternative therapies may now derive from the current findings showing that ER molecules occur not only in the nucleus of the cell, but also in association with the surface membranes of human breast cancer cells. Moreover, these ER may interact with membrane HER-2 growth factor receptors. It is known that expression of HER-2 receptors occurs in many human breast cancers, and the enzyme activity of HER-2 may play a role in ER activation even in the absence of estrogen. If active cross-talk between ER and the HER-2 growth factor receptor occurs and leads to promotion of cancer growth, this signaling axis may offer a new target for therapeutic intervention. Since overexpression of HER-2 in human breast cancers is associated with the failure of antiestrogen therapy in the clinic, understanding the biologic basis of the association between membrane ER and HER-2 receptors may help to improve decisions on patient management and to increase patient survival.

In the present work, we have made good progress in ascertaining the existence and nature of receptors for estrogen in surface membranes of human breast cancer cells. We have begun to assess the role of membrane ER in promoting growth of breast cancers. In challenging the dogma of estrogen action exclusively via an intracellular receptor, this work may lead to the development of previously unsuspected, less toxic antitumor therapies targeted to human breast cancer cells.

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Steroid Hormone Receptors in Target Cell Membranes

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Numerous reports of rapid steroid hormone effects in diverse cell types cannot be explained by the generally prevailing theory that centers on the activity of hormone receptors located exclusively in the nucleus. Cell membrane forms of steroid hormone receptors coupled to intracellular signaling pathways may also play an important role in hormone action. Membrane-initiated signals appear to be the primary response of the target cell to steroid hormones and may be prerequisite to subsequent genomic activation. Recent dramatic advances in this area have intensified efforts to delineate the nature and biologic roles of all receptor molecules that function in steroid hormone-signaling pathways. This work has profound implications for our understanding of the physiology and pathophysiology of hormone actions in responsive cells and may lead to development of novel approaches for the treatment of many cell proliferative, metabolic, inflammatory, reproductive, cardiovascular, and neurologic defects.

Key Words: Steroid hormone action; plasma membrane; receptor.

Introduction

The broad physiologic effects of steroid hormones in the regulation of growth, development, and homeostasis have been known for decades. Often, these hormone actions culminate in altered gene expression (1), which is preceded by nutrient uptake and other preparatory changes in the synthetic machinery of the cell (2). Owing to certain homologies of molecular structure, specific receptors for steroid hormones, vitamin D, retinoids, and thyroid hormone are often considered a receptor superfamily. The actions of ligands in this steroid receptor superfamily are commonly postulated to be mediated by receptors in the cell nucleus. On binding ligand, nuclear receptors associate with target

genes and permit selective transcription. This genomic mechanism is generally slow, often requiring hours or days before the consequences of hormone exposure are evident. However, steroids also elicit rapid cell responses, often within seconds. The time course of these acute events parallels that evoked by peptide agonists, lending support to the conclusion that they do not require precedent gene activation (2–5). Rather, many rapid effects of steroids, which have been termed *nongenomic*, appear to be owing to specific recognition of hormone at the cell membrane. Although the molecular identity of binding sites remains elusive and the signal transduction pathways require fuller delineation, there is mounting evidence that steroid action is initiated by plasma membrane receptors.

A current challenge is to determine the relation of rapid responses to steroid hormones to intermediate and long-term effects. Some questions that arise in this context include the following: Is specific membrane binding responsible merely for cellular entry of the hormone? Do plasmalemmal receptors escort ligand to the nucleus? Are the membrane binding sites coupled to rapid signal transduction systems that also act in concert with nuclear transcription factors? Are the membrane receptors identical to nuclear receptors, modified forms, or entirely different entities? This review explores these important issues. In preparing this work, more than 1200 references providing significant evidence for rapid steroid actions and for membrane forms of steroid receptors were identified. Only a fraction of these citations can be presented here, and the reader is referred to several recent reviews in this area (3–7).

Estrogens

As with other steroid hormones, biologic activities of estrogen in breast, uterus, and other tissues are considered to be fully mediated by a specific high-affinity receptor in cell nuclei. Estrogens are accumulated and retained in responsive cells, and it has been commonly assumed that the steroid diffuses passively to intracellular receptors. However, estradiol is a lipophilic molecule that partitions deep within the hydrocarbon core of lipid bilayer membranes, even those devoid of relevant receptors (3). Several investigations now demonstrate that steroid hormones enter target cells by a membrane-mediated process that is saturable

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Table 1
Brief Chronology of Selected Reports Documenting Occurrence and Activity of Membrane Steroid Hormone Receptors^a

Steroid	Year	Observation	Reference
Estradiol	1967	Elevation of uterine cAMP by estrogen within seconds	11
	1975	Rapid endometrial cell calcium mobilization by estrogen	9
Corticosterone	1976	Binding to plasma membranes of rat liver	108
	1977	Effects on electrical activity of neurons	20
Cortisol	1978	Specific plasma membrane binding sites for estrogen	16
Progesterone	1978	Electrophysiologic effects on neurons	21
Estradiol	1979	Induction of oocyte maturation by steroid linked to a polymer	29
	1980	Increased proliferation of cells with membrane ER	17
Vitamin D	1981	Molecular properties of ERs in liver plasma membrane	13
Progestin	1982	Rapid intestinal cell calcium uptake	109
	1982	Specific binding to oocyte surface and role in meiotic maturation	30
Corticosterone	1983	Steroid receptor of 110 kDa on oocyte surface by photoaffinity labeling	31
Estradiol	1983	Binding to synaptic plasma membranes	50
	1984	Increase in density of microvilli at endometrial cell surface within seconds	112
Thyroid hormone	1985	Primary internalization of ER in endometrial plasma membrane vesicles	104
Estradiol	1986	Characterization of plasma membrane binding sites	47
	1986	High-affinity binding sites in breast cancer cell plasma membranes	26
Glucocorticoid	1987	Altered breast cell membrane potential, density of microvilli within seconds	110
Vitamin D	1989	Correlation between membrane receptor and apoptosis in lymphoma cells	53
Thyroid hormone	1990	Rapid activation of phospholipase C (PLC) in rat intestine	5,14
	1991	Activation of calcium channels in osteoblasts	63
Progesterone	1990	Rapid induction of glucose uptake	42
Corticosterone	1991	Stimulation of calcium influx in human sperm	33
	1991	Calcium uptake mediated by sperm cell surface-binding sites	34
Aldosterone	1993	Action at plasma membrane of human sperm	51
Glucocorticoid	1993	Correlation of neuron membrane receptors with behavior in newts	111
Estradiol	1994	Rapid effects on Na ⁺ /H ⁺ exchange	54
	1994	Antigenic similarity between membrane and intracellular receptors	90
Vitamin D	1994	Binding and stimulation of HER-2 membrane receptor	12
Aldosterone	1995	Activation of adenylate cyclase signaling pathways	88
Estradiol	1995	Isolation of a plasma membrane receptor from chick intestine	86
	1995	Identification of membrane receptor in human lymphocytes	7,78
Androgen	1997	Membrane receptor with antigenic identity to nuclear receptor	36
Estradiol	1997	Greater nongenomic responses of membrane receptor-enriched neural cells	14
	1997	Rapid increase in cytosolic Ca ⁺⁺ in Sertoli cells	81
Vitamin D	1998	Membrane action and PLC regulation	65
Estradiol	1999	Isolation of membrane binding-proteins from rat brain	10
	1999	Blocking of hormone activation of PKC by antibody to membrane receptor	94
Androgen	2000	Rapid Ca ⁺⁺ mobilization required for activation of MAPK	80
Progesterone	2000	Rapid actions in neurons from ERα knockout mice	25
Vitamin D	2000	Reduction of membrane ER expression by antisense to nuclear ER	37
Estradiol	2000	Membrane and nuclear ERα, and ERβ, each expressed from single transcript	85
	2000	Activation of G-proteins, IP ₃ , adenylate cyclase, and MAPK by membrane ER	89
Androgen	2000	Rapid activation of MAPK pathway in prostate	79
Progesterone	2000	Cloning and expression of binding protein from liver microsomal membrane	113
Vitamin D	2000	Ligand-induced nuclear translocation of plasma membrane receptor	114
Estradiol	2000	Surface receptor in endothelial cells recognized by monoclonal ERα antibody	114
	2000	Interaction of ERα with regulatory subunit of phosphatidylinositol-3-OH kinase	resulting in prolactin gene expression in pituitary cells

^aMore than 1200 publications on membrane steroid receptors have appeared in the past 30 yr. Of these, only representative examples are listed here. The potential roles of alternate (25) or variant (56) forms of steroid hormone receptors and other membrane-signaling molecules (90,94) remain to be clarified.

and temperature dependent (3,8). Moreover, it is well established that estrogen can trigger in target cells rapid surges in levels of intracellular messengers, including calcium (9, 10) and cyclic adenosine monophosphate (cAMP) (11,12), as well as activation of mitogen-activated protein kinase

(MAPK) (13) and phospholipase (14) (Table 1). These data have led to a growing consensus that the traditional genomic model of estrogen action does not explain the rapid effects of estrogens and must be expanded to include membrane receptors as a component of cell signaling (2–7,15).

The first unequivocal evidence for specific membrane-binding sites for estradiol- 17β (E_2) was reported in 1977 (16). Intact uterine endometrial cells equipped with estrogen receptor (ER), but not ER-deficient control cells, bound to an inert support with covalently linked E_2 . In addition, target cells that bound could be eluted selectively with free hormone, and cells so selected exhibited a greater proliferative response to estrogens than cells that did not bind (17,18). Further investigations have continued to provide compelling evidence for the occurrence of a plasma membrane form of ER and support for its role in mediating hormone actions (3) (Table 1).

Selye (19) first demonstrated that steroids at pharmacologic concentrations elicit acute sedative and anesthetic actions in the brain. However, electrical responses to *physiologic* levels of E_2 with rapid onset have since been reported in nerve cells from different brain regions (4,20,21). Similarly, certain vasoprotective effects of estrogen appear attributable to membrane receptors (15,22). Estrogen-induced release of uterine histamine *in situ* has long been associated with rapid enhancement of the microcirculation by a process that excludes gene activation (2). Reinforcing these observations are new data detailing the role of nitric oxide (NO) in vascular regulation by estrogen. Normal endothelium secretes nitric oxide, which relaxes vascular smooth muscle and inhibits platelet aggregation. Estrogens elicit abrupt liberation of NO by acute activation of endothelial NO synthase without altering gene expression, a response that is fully inhibited by concomitant treatment with specific ER antagonists (23). This estrogenic effect may be mediated by a receptor localized in caveolae of endothelial cell membranes (24). Such observations require extension, because several independent cell-signaling complexes that appear to participate in signal transduction to the nucleus also associate with caveolar structures (2,3,22).

Estrogen deficiency is associated with significant bone loss, and research on the potential role of membrane ERs in regulating bone mass has increased. Evidence for membrane-binding sites and acute effects of estrogen with an onset within 5 s has been reported in both osteoblasts and osteoclasts (5,13). The effects of estrogens on bone homeostasis also appear to involve rapid activation of MAPK (13), as has also been demonstrated in certain other target cells (10,15,25).

When exposed to E_2 conjugated to fluorescein-labeled bovine serum albumin (BSA), human breast cancer cells exhibit specific surface staining (7,26). Since E_2 -BSA is considered membrane impermeant, these conjugates, developed primarily for use as immunogens and for affinity purification of nuclear ERs, have also been used to assess the membrane effects of estrogen. However, in light of the fact that E_2 -BSA is unstable in solution, especially in the presence of cells and their enzymic products, and releases measurable amounts of free steroid (27), data relying only on the use of estradiol conjugates to test for membrane effects

of steroids need especially careful scrutiny. It is clear that more stable, cell-impermeant derivatives of estradiol should be developed for evaluating membrane receptors.

Progestogens and Androgens

As documented for estrogens, several physiologic effects of progestogens and androgens appear to be regulated, in part, by membrane receptors. Progesterone controls components of reproductive function and behavior. Some of these activities are mediated by interaction with neurons in specific brain regions, and membrane effects appear to be important in this process (4,28). Meiosis in amphibian oocytes is initiated by gonadotropins, which stimulate follicle cells to secrete progesterone. The progesterone-induced G₂/M transition in oocytes was among the first convincing examples of a steroid effect at plasma membrane, since it could be shown that exogenous, but not intracellularly injected, progesterone elicited meiosis and that many progesterone-stimulated changes occurred even in enucleated oocytes (29–32). Moreover, this process may be related to progesterone-induced increments in intracellular Ca⁺⁺ and release of diacylglycerol species that elicit a cascade of further lipid messengers (32).

Progesterone elicits rapid effects on membrane receptors, second messengers, and the acrosome reaction in human sperm (33–35). Assay of acute sperm responses to progesterone in subfertile patients is highly predictive of fertilizing capacity (35). Effects of the steroid, present in the cumulus matrix surrounding the oocyte, appear to be mediated by elevated intracellular Ca⁺⁺, tyrosine phosphorylation, chloride efflux, and stimulation of phospholipases, effects attributed to activation of a membrane-initiated pathway. Indeed, two different receptors for progesterone, apparently distinct from genomic ones, have been identified at the surface of human spermatozoa (35); nevertheless, a monoclonal antibody (MAb) against the steroid-binding domain of human *intracellular* progesterone receptor (PR) inhibits progesterone-induced calcium influx and the acrosome reaction in sperm (35).

As with estrogens and progestogens, androgens promote a rapid increase in cytosolic Ca⁺⁺ in their cellular targets (36). Other effects of androgens that are not attributable to genomic activation include acute stimulation of MAPK in prostate cancer cells (37). The androgen, 5 β -dihydrotestosterone, induces vasodilation of aorta, which may be owing to direct action of the steroid on membranes of smooth muscle cells leading to modulation of calcium channels (38). In osteoblasts, membrane receptors for androgen appear to be coupled to phospholipase C (PLC) via a pertussis toxin-sensitive G-protein that, after binding testosterone, mediates rapid increments in intracellular calcium and inositol triphosphate (IP₃) (39). Of note, Benten et al. (40) report that testosterone elicits Ca⁺⁺ mobilization in macrophages that lack intracellular androgen receptor (AR). These cells

express an apparent G-protein-coupled AR at the cell surface that undergoes agonist-induced internalization.

Thyroid Hormones

Thyroid hormones are well known to regulate energy expenditure and development, and membrane-initiated effects may contribute to these responses. Triiodothyronine (T_3) rapidly stimulates oxygen consumption and gluconeogenesis in liver (41). T_3 also promotes an abrupt increase in uptake of the glucose analog, 2-deoxyglucose, in responsive tissues by augmenting activity of the plasma membrane transport system for glucose (42). In rat heart, T_3 elicits a positive inotropic effect, increasing left ventricular peak systolic pressure, as early as 15 s after hormone (43). In each tissue investigated, alterations in intracellular Ca^{++} induced by thyroid hormone appear to modulate signal transduction to the cell interior (41–44).

Membrane-initiated effects of T_3 have been documented in bone cells by means of inositol phosphate signaling (45), and in brain through calcium channel activation (46). T_3 can also influence other cell processes, including the exocytosis of hormones and neurotransmitters (46), rapid effects that may be attributable to mediation by membrane receptors (44). Although uptake of T_3 can occur concomitantly with receptor-mediated endocytosis of low-density lipoprotein, and likely accompanied by carrier proteins, uptake of T_3 itself has also been reported to occur in numerous tissues by means of a high-affinity, stereospecific, and saturable process (45,47,48), as found for steroid hormones (3,8).

Glucocorticoids

In addition to their long-established effects on mobilization of energy sources by promoting catabolism and the induction of enzymes involved in gluconeogenesis, glucocorticoids have profound effects on neuron signaling and on induction of apoptosis in lymphocytes, phenomena that appear to be membrane-initiated events. Kelly et al. (21) found that glucocorticoids rapidly altered neuron-firing patterns, and many studies have verified these effects (4,6,28). These molecular events lead to glucocorticoid modulation of specific brain functions, such as the rapid response of hypothalamic somatostatin neurons to stress (49). Such abrupt changes in neuron polarization are reinforced by findings of specific, saturable binding of corticosterone to neuron membranes (50,51). Specific, high-affinity corticosterone binding to calf adrenal cortex plasma membrane is also identified by use of the biologically active radioligand [^3H]corticosterone (52).

Glucocorticoids also play an important role in the regulation of immune function and inflammation. In lymphoproliferative diseases, glucocorticoids are in wide use as therapeutic agents, but the cellular mechanism leading to the therapeutic effect remains unclear. In several studies using both cell lines and freshly prepared leukemia or lym-

phoma cells, the presence of a membrane receptor for glucocorticoids has been implicated in modulating apoptosis and cell lysis (7,53–55). Moreover, in lymphocytes, the membrane-binding site is antigenically related to the intracellular glucocorticoid receptor (iGR) and may be a natural splice variant form of the intracellular receptor (7,55,56). A potential parallel to the ER transfected in Chinese hamster ovary (CHO) cells (25) is evident.

Aldosterone and Digitalis-Like Steroids

Beyond its classic functions of promoting renal reabsorption of sodium and excretion of excess potassium, aldosterone enhances sodium absorption from colon and urinary bladder. In each tissue, the mineralocorticoid effect is owing to enhanced activity of amiloride-sensitive sodium channels. Aldosterone rapidly augments Na^+/H^+ exchange (6,57). This function is Ca^{++} - and protein kinase C (PKC)-dependent but independent of nuclear receptor activation, transcription, and protein synthesis (6,58). Similarly, “non-genomic” action of aldosterone has also been reported to underlie its acute effects on cardiac function and on sodium transport in vascular smooth muscle cells (6,58).

Digitalis-like compounds are often forgotten members of the steroid superfamily. These plant-derived agents elicit inotropic and chronotropic effects on the heart but also affect many other tissues. Endogenous steroid ligands, termed *digitalis-like* or *ouabain-like* factors, have been found in sera of humans and other animals with blood volume expansion and hypertension (59,60) and may be released from adrenal cortex (60). These ligands elicit inhibition of membrane-associated Na^+/K^+ -ATPase, likely the principal receptor for these agonists. It is notable that the steroid-binding domain of Na^+/K^+ -ATPase and that of nuclear hormone receptors share significant amino acid sequence homology (61). In addition to membrane actions of these compounds on Na^+/K^+ -ATPase, ouabain-induced hypertrophy in myocytes is accompanied by promotion of Ca^{++} flux and initiation of protein kinase-dependent pathways leading, in turn, to specific changes in transcription and altered expression of early response- and late-response genes (62). Thus, the biologic effects of digitalis-like compounds, long considered the exception to the concept of exclusive genomic influence, may render them more closely integrated with the steroid hormone superfamily than was previously recognized.

Vitamin D Metabolites

Membrane-initiated effects of the seco-steroid hormone, 1,25-dihydroxyvitamin D_3 (1,25[OH] $_2\text{D}_3$), are well documented in bone and cartilage. In osteoblasts, Caffrey and Farach-Carson (63) elucidated possible connections between rapid effects of 1,25(OH) $_2\text{D}_3$, requiring milliseconds to minutes, and longer-term effects owing to gene expression. Their laboratory was the first to show activa-

tion of calcium channels by $1,25(\text{OH})_2\text{D}_3$ (63). Calcium, which can signal gene expression through multiple pathways, promotes key phosphorylation events in certain bone proteins (5). Osteoblasts exhibit rapid changes in IP_3 and diacylglycerol in response to vitamin D metabolites via activation of PLC (5,14). Other bone cells with rapid responses to vitamin D metabolites include osteosarcoma cells and chondrocytes (5,64). The latter system is particularly intriguing because chondrocytes elaborate matrix vesicles that appear critical in bone mineralization. The matrix vesicles, which lack nuclei, exhibit specific, saturable binding of $1,25(\text{OH})_2\text{D}_3$, especially when derived from growth zone chondrocytes (65).

Other rapid effects of vitamin D occur in a variety of cell types. Muscle cells respond within seconds to $1,25(\text{OH})_2\text{D}_3$ via several mediators that alter cardiac output in some instances, while acute activation of calcium channels in skeletal muscle promotes contraction (5,66). Of note, in lymphoproliferative disease, $1,25(\text{OH})_2\text{D}_3$ appears to prime monocytic leukemia cells for differentiation through acute activation or redistribution of PKC, Ca^{++} , and MAPK (5,67). In pancreas and intestine, activation of membrane-associated signaling pathways results in vesicular exocytosis. Pancreatic β -cells respond to $1,25(\text{OH})_2\text{D}_3$ with enhanced intracellular Ca^{++} coupled to increased insulin release (68). In intestine, $1,25(\text{OH})_2\text{D}_3$ stimulates exocytosis of transported vesicular calcium and phosphate. These cellular events may be related to vitamin D-promoted alterations in the levels of α -tubulin (5), thereby influencing assembly of microtubules and possibly providing a means for vectorial transport of absorbed ions. Several signal transduction pathways have been found to respond rapidly to exogenous $1,25(\text{OH})_2\text{D}_3$, including activation of protein kinases and promotion of abrupt increments in Ca^{++} , but integration of these signaling cascades with the physiologic response of enhanced ion absorption remains to be established (5,68,69).

Investigations with vitamin D congeners have recently indicated the potential hormonal nature of $24,25(\text{OH})_2\text{D}_3$, once thought to represent merely the inactivation product of precursor $25(\text{OH})\text{D}_3$. Acute effects of $24,25(\text{OH})_2\text{D}_3$ have been observed in bone cells and in intestine; $24,25(\text{OH})_2\text{D}_3$ also inhibits rapid actions of $1,25(\text{OH})_2\text{D}_3$ (5). This may explain why abrupt effects of $1,25(\text{OH})_2\text{D}_3$ often fail to be observed *in vivo* (70): normal, vitamin D-replete subjects have endogenous levels of $24,25(\text{OH})_2\text{D}_3$ sufficient to inhibit acute stimulation of calcium transport by $1,25(\text{OH})_2\text{D}_3$, thus providing a feedback regulation system (69).

Retinoids

Retinoic acid exerts diverse effects in the control of cell growth during embryonic development and in oncogenesis. It is widely considered that effects of retinoids are mediated through nuclear receptors, including those for retinoic acid as well as retinoid X receptors (1). However,

other retinoid response pathways appear to exist, independent of nuclear receptors (71). Cellular uptake of retinol ('vitamin A') may involve interaction of serum retinol-binding protein with specific surface membrane receptors followed by ligand transfer to cytoplasmic retinol-binding protein (72). In this regard, targeted disruption of the gene for the major endocytotic receptor of renal proximal tubules, megalin, appears to block transepithelial transport of retinol (73). It is noteworthy that megalin may also be implicated in receptor-mediated endocytosis of $25(\text{OH})\text{D}_3$ in complex with its plasma carrier (74). In addition, retinoic acid binds mannose-6-phosphate (M6P)/insulin-like growth factor-2 (IGF-2) receptor with moderate affinity and appears to enhance its receptor activity (75). M6P/IGF-2 receptor is a membrane glycoprotein that functions in binding and trafficking of lysosomal enzymes, in activation of transforming growth factor- β , and in degradation of IGF-2, leading to suppression of cell proliferation. The concept of multiple ligands binding to and regulating the function of a single receptor is relatively novel but has important implications for modulating and integrating the activity of seemingly independent biologic pathways.

Properties of Membrane Receptors for the Steroid Superfamily

Despite renewed interest in membrane steroid receptors, the physical identity of receptors with high binding affinity for ligand remains elusive. Isolation and structural characterization of these molecules remains to be accomplished. They may be known membrane components (e.g., enzymes, ion channel subunits, receptors for nonsteroid ligands), with previously unrecognized binding sites for steroids, new forms of steroid hormone receptors, "classic" receptors complexed with other membrane-associated proteins, or truly novel membrane proteins.

Estrogens and Progestogens

Efforts to isolate and purify membrane receptors that mediate rapid effects of steroids are under way in several laboratories (Table 2). Early work on purification of ER from uterus and liver plasma membranes suggested that it was a protein species with high-affinity, saturable binding specific for estradiol-17 β (16,18). The molecular size of solubilized receptor was in the range of intracellular ER (18,76). Other work to isolate plasma membrane estrogen-binding proteins identified the 67-kDa species characteristic of nuclear receptor, but additional proteins of variant size ranging from 28 to 200 kDa were also revealed (77). To determine whether membrane ER had antigenic homology with nuclear ER, Pappas et al. (78) used antibodies prepared to different functional epitopes of intracellular receptor and demonstrated surface labeling in nonpermeabilized rat pituitary cells by confocal scanning laser microscopy. Recent work by Russell et al. (79) has demonstrated, by means

Table 2
Representative Examples of Physical Properties of Membrane-Associated Receptors for Ligands of Steroid Hormone Superfamily^a

Ligand	MW (kDa)	K_d (M)	Binding capacity (fmol/mg protein)	Homology with nR	Tissue	Reference
Estradiol	51–78 ^b	2.8×10^{-10}	526	ND	Rat hepatocytes	18
	105–148 ^c					
	11–67	3.6×10^{-10}	370	ND	Rabbit uterus	77
Progesterin	67			Yes	CHO cell (ER transfected)	25
	110	5×10^{-7}		ND	Amphibian oocyte	30
	110	1×10^{-6}		ND		31
Vitamin D	28.56	6.9×10^{-8}	Variable	ND	Porcine liver	84
	65	7×10^{-10}	240	No	Chick intestine	88
		1.7×10^{-11}	124	No	Rat growth chondrocytes	65
		2.8×10^{-11}	100	No	Rat resting chondrocytes	
Aldosterone	36	1×10^{-8}		ND	Rat osteoblast-like cells	87
	50	1.1×10^{-8}	350	No	Pig liver	86
Glucocorticoids		1×10^{-7}		ND	Rat synapses	50
	97–150	2.4×10^{-7}	384	Yes	S-49 lymphoma cells	55
		5.1×10^{-10}		ND	Amphibian synapses	51
Thyroid hormone	145	2×10^{-9}	320	No	Human placenta	47
		6×10^{-10}		ND	Rat myoblasts	48

^aOnly representative examples of steroid-binding membrane macromolecules are presented here. Please refer to text for additional references. Homology of membrane macromolecules to nuclear receptor forms (nR) is noted; MW, apparent molecular weight; ND, not determined.

^bHigh salt (0.4 M KCl).

^cLow salt (0.01 M KCl).

of monoclonal anti-ER α , that human endothelial cells possess surface-binding sites for estrogen (see Table 1). In evaluating the source and distribution of membrane ER, target cells with expression of ER α were treated with antisense oligonucleotide to nuclear ER α to suppress expression of receptor protein (80). This approach significantly reduced expression of membrane as well as nuclear forms of ER. Using an alternate method to assess receptor origin, Razandi et al. (25) transfected cDNA for ER α and ER β into CHO cells, which do not normally express ER. The transfections resulted in ER expression in both nuclear and membrane fractions, suggesting that membrane and nuclear ER are derived from a single transcript. In addition, both ER α and ER β were expressed in membranes, and both receptors were capable of activating G-proteins, MAPK, as well as DNA synthesis (25). In related studies, the acute stimulation of endothelial nitric oxide synthase (eNOS) by estrogen was reconstituted in COS-7 monkey kidney cells cotransfected with ER α and eNOS, but not by transfection with eNOS alone (23).

Binding molecules for estrogen and progesterone, comprising several molecular species, were isolated from brain synaptosomes by affinity chromatography and characterized by electrophoresis and Western blot (15,81). Microsequencing of one E $_2$ -binding protein indicated that the high-affinity site corresponds to the subunit of an ATPase/ATP synthase. In addition, some studies suggest that estrogen

bound to sex hormone-binding globulin, a plasma protein, also binds with specificity to membrane sites recognizing the liganded transport protein (82). These transport-protein interactions promote cAMP generation via the intermediacy of G-proteins. However, further characterization of receptors for such steroid:protein complexes is not available, and it must be recalled that estrogen is in noncovalent association with its plasma protein carrier and dissociates readily therefrom (83).

Binding of progesterone to plasma membrane of amphibian oocytes is specific, saturable, and temperature dependent (31,32). Photoaffinity labeling with the synthetic progestin [3 H]-R5020, followed by gel electrophoresis, revealed progestin binding to both 80- and 110-kDa proteins in oocyte cytosol, whereas only the 110-kDa R5020-binding protein was present in oocyte plasma membrane. A progesterone-binding protein (msPR) was identified in crude microsomal, rather than purified plasmalemmal, membranes from porcine liver (84,85). On solubilization, a moderate-affinity site with a dissociation constant (K_d) of 69 nM was found, but, after further purification, affinity decreased to K_d of 228 nM. The final fraction contained two novel peptides of 28 and 56 kDa. Expression of msPR-cDNA in CHO cells led to slightly increased progesterone binding in microsomes, and administration of an antibody against msPR reduced rapid progesterone-initiated Ca $^{++}$ increases in sperm (85). Whether this work represents the first successful cloning

and expression of a steroid receptor associated with cell membranes will have to await confirmation. However, Falkenstein et al. (85) suggest that the native *plasma* membrane PR may actually be an oligomeric protein complex of about 200 kDa, composed only in part by 28- and 56-kDa peptides.

Glucocorticoids, Aldosterone, and Vitamin D

Progress has been made in the isolation and characterization of plasma membrane receptors for glucocorticoids, aldosterone, and $1,25(\text{OH})_2\text{D}_3$, although at this writing, evidence of cloning of the cDNA for any of these proteins is lacking. The membrane glucocorticoid receptor (mGR) was purified from lymphoma cells by immunoaffinity binding with an MAb coupled to Sepharose-4B; the protein displayed properties similar to iGR (55). Scatchard analysis of mGR yielded a K_d of 239 nM and B_{\max} of 384 fmol/mg of protein, representing a somewhat higher number of binding sites but a lower affinity than that of the iGR. Peptide maps revealed some sequences that were unique to the membrane form (55,56). Further data suggest that the mGR in lymphoma cells is a transcript variant of the iGR (56) (Table 2). Properties of the aldosterone membrane receptor have been analyzed by means of [^{125}I]-aldosterone photo-affinity labeling. The protein has an apparent molecular mass of 50 kDa and appears to be distinct from intracellular receptor (86).

The pursuit of membrane receptor for $1,25(\text{OH})_2\text{D}_3$ (pmVDR) by affinity isolation has been hampered by the fact that most ligand derivatives lack sufficient binding activity. Nevertheless, work by Baran et al. (87) indicates that the vitamin D analog, [^{14}C]- $1\alpha,25$ -dihydroxyvitamin D₃ bromoacetate, does exhibit a moderate degree of specific binding to a 36-kDa protein in plasma membranes of rat osteoblast-like cells. Using sequence determination and Western blot, the labeled membrane protein was identified as annexin II, part of a family of membrane-binding proteins previously implicated in the regulation of Ca^{++} signaling, tyrosine phosphorylation, and apoptosis. Partially purified plasma membrane proteins and purified annexin II exhibited specific and saturable binding for [^3H]- $1\alpha,25(\text{OH})_2\text{D}_3$, and antibodies to annexin II inhibited [^{14}C]- $1\alpha,25(\text{OH})_2\text{D}_3$ bromoacetate binding to plasma membranes and also inhibited hormone-induced increases in intracellular calcium in osteoblast-like cells. Hence, these initial results (87) suggest that annexin II may serve as a receptor for rapid actions of $1,25(\text{OH})_2\text{D}_3$ in rat osteoblast-like cells, but it is not known if this receptor system functions in other cell types. In independent studies, classic biochemical strategies, coupled with analyses of specific binding, were used to isolate the vitamin D membrane receptor (pmVDR) from intestinal epithelium of chicks (88). Basal-lateral membranes were solubilized with detergent and subjected to ion-exchange and gel filtration chromatography. Binding activity eluted with a protein of 65 kDa, with a K_d of 0.7 nM

(88). A highly specific antibody toward plasma membrane VDR failed to recognize the nuclear receptor in Western analyses. On the other hand, a commercially available MAb generated against the "classic" nuclear receptor reacted with many proteins in nuclear fractions of chick intestine, including a band that comigrated with authentic recombinant protein, but did not detect VDR in basolateral membranes (89). Antibody to the plasma membrane receptor, but not to the nuclear receptor, blocked hormonal activation of PKC. The 65-kDa protein was also observed to bind the affinity ligand, [^{14}C]- $1\alpha,25$ -dihydroxyvitamin D₃ bromoacetate, and labeling was diminished in the presence of excess nonradioactive ligand (89). Electron microscopic studies of duodena vascularly perfused with control media, $1,25(\text{OH})_2\text{D}_3$, or $24,25(\text{OH})_2\text{D}_3$ followed by immunohistochemical staining revealed that $1,25(\text{OH})_2\text{D}_3$, but not control media or $24,25(\text{OH})_2\text{D}_3$, resulted in dramatically enhanced nuclear localization of the putative membrane receptor (89).

Varied Forms of Steroid Hormone Receptors in Plasma Membranes

Collectively, current findings suggest that membrane receptors for steroid hormones are, in certain instances, transcriptional copies (estrogen) or variants (glucocorticoids) of nuclear receptors and, in other instances, products apparently unrelated to intracellular receptors (aldosterone and vitamin D). There is evidence for alternatively spliced transcripts of several steroid receptors, and these variant receptors give rise to proteins of different molecular size and, possibly, modified properties (56). Membrane insertion of receptors in primary transcript form would likely require one or more hydrophobic regions, and post-translational modification of receptor protein leading to cell membrane targeting may also occur, including phosphorylation, glycosylation, and addition of lipid anchors or other modifications, such as palmitoylation or myristoylation. Surface steroid hormone receptors may also be part of a multimeric complex including a "classic" nuclear receptor but bound to as-yet-unidentified transmembrane proteins and coupled to membrane-associated signaling molecules (3,7,15,79). Alternatively, plasma membrane receptors for steroids may have several common structural features with, but may be distinct from, the intracellular steroid hormone receptors (88,89). In the case of retinoic acid and estradiol, binding to known membrane proteins, such as M6P/IGF-2 receptor (75) or HER-2 receptor (90), respectively, may modulate some ligand effects. Progesterone appears to interact directly with oxytocin receptor, a G-linked protein at the cell surface, and inhibits some functional effects of oxytocin signaling, thus suppressing uterotonic activity of oxytocin (91). Progesterone congeners also bind with moderate affinity to γ -aminobutyrate type A (GABA_A) receptors that comprise ligand-gated ion channel complexes (4,28). Absence of the γ -subunit of GABA_A receptor in appropriate knockout mice results in a significant decrease in

sensitivity to neuroactive steroids such as pregnanolone (92). Similarly, acute vascular relaxation induced by pharmacologic levels of E₂ may be mediated by its binding to the regulatory subunit of Maxi-K channels in membranes (93), thus supporting the view that some effects of steroids, at least at high micromolar concentration, may be mediated by known membrane receptors with previously unrecognized steroid-binding sites.

Using ER α gene knockout (ERKO) mice, Gu et al. (94) showed that rapid actions of estradiol at 50 nM on kainate-induced currents in hippocampal neurons still occur, and the effect is not inhibited by ICI 182,780, a pure antagonist of hormone binding to both ER α and ER β . These investigators suggest that a distinct estrogen-binding site exists in neurons and appears to be coupled to kainate receptors by a cAMP-dependent process. However, it is important to note that alternatively spliced forms of ER α (95), as well as ER β (96), can occur in ERKO mice, thus complicating the interpretation of these results. Moreover, uterine tissues of ovariectomized ERKO mice exhibit 5–10% of the estradiol binding present in wild-type uteri (95,97), and the significance of these residual estrogen-binding sites in ERKO target cells is unclear. Nonetheless, further development of double ER α and ER β gene knockouts and perfection of this new technology should prove important in deciphering the contribution of "classic" and novel receptor forms in hormone action.

In future work, it will be important to pursue isolation and characterization of constituent proteins from homogeneous plasma membranes prepared in the presence of proteinase inhibitors (18,76,98). Verification of their purity should be confirmed by use of a balance sheet for enzyme or other membrane markers (18,76). Screening for activity of receptor would benefit from the use of independent approaches, such as ligand binding with radio- or photoaffinity-labeled steroids and immunoassay directed toward known intracellular receptors (15,31,55,78,86). These several approaches may detect membrane receptors originating from a transcript other than that of intracellular receptor. As with the mixed steroid hormone-binding protein systems known to occur within cells and in their extracellular fluids, it may well be that multiple forms of receptor proteins for steroids coexist in plasma membranes, thus complicating efforts to isolate and characterize the individual binding species in this cell compartment. Our efforts to understand ligand-receptor interactions are often limited by simplistic "lock-and-key" models that may not accurately reflect the true state of complex molecular signaling cascades. Study of the molecular organization of several neurotransmitter receptor families has already shown that extraordinary biologic variability occurs, with multiple "keys" and multiple "locks" sometimes involved in ligand-receptor recognition (99). We must consider the existence of similar high-affinity, but possibly multivalent and multifunctional, receptors in the steroid hormone superfamily (75,91–93).

Perspectives

Ever since the discovery of chromosomal puff induction by ecdysone, cell regulation by steroid hormones has focused primarily on a nuclear mechanism of action. However, even the venerable steroid hormone ecdysone elicits rapid membrane effects that may facilitate later nuclear alterations (100). Indeed, membrane-initiated responses appear to be the cell's earliest response to steroids and may be prerequisite to subsequent genomic responses (2,3,7,10; see also Fig. 1). Coupling of surface membrane, cytoplasmic, and nuclear responses may offer a progressive, ordered expansion of initial signal. Accordingly, the terms *genomic* and *nongenomic* may not accurately define such a response continuum (101). Future investigations should focus on potential interactions of membrane and nuclear steroid receptors that may promote activation of transcription and other specific hormonal responses. Molecular details of cross-communication between steroid and peptide receptors are also beginning to emerge (3,98), and membrane steroid receptors may be in a pivotal location to promote convergence among diverse signaling pathways (Fig. 1). Indeed, the consequences of steroid hormone recognition at the outer cell membrane of target, but not nontarget, cells are shared by numerous other classes of regulatory molecules (cf. ref. 102), including peptide hormones, neurotransmitters, drugs, plant lectins, mitogens, and antibodies (3). Although the agonists are manifold, the signaling mechanisms are few. Primary signal recognition at the surface would be fleeting, but the mutual specificities and affinities are high, and thus sufficient for setting the appropriate signal transduction chain in motion. However, until the current surge of renewed focus on this problem, identification of these instantaneous triggering interactions for steroid hormones has accumulated relatively slowly, having been limited by technical and microanalytic barriers that are now being surmounted.

Ligand-receptor interactions depend on an extensive array of extracellular and intracellular partners to localize to membrane microdomains, recruit signaling molecules, and trigger intracellular signaling pathways. As the consequences of surface interactions are analyzed in greater depth, it will be important to evaluate further the biologic role of rapid internalization of steroid-binding sites from plasma membranes via endocytotic-lysosomal pathways (2,3,88,101,103–105). These membrane-initiated events may involve cytostructural elements or scaffold proteins that contribute to signal propagation to the nucleus and the nuclear-protein matrix (2,101,104–107; Fig. 1). Thus, antibodies specific to intestinal membrane VDR reveal a vitamin D-induced redistribution of membrane receptor, a protein that appears distinct from intracellular receptor, to the nucleus within 5 min of binding ligand (89). It is unknown whether the membrane receptor has inherent DNA- or coregulator-binding capacity to alter transcription; alter-

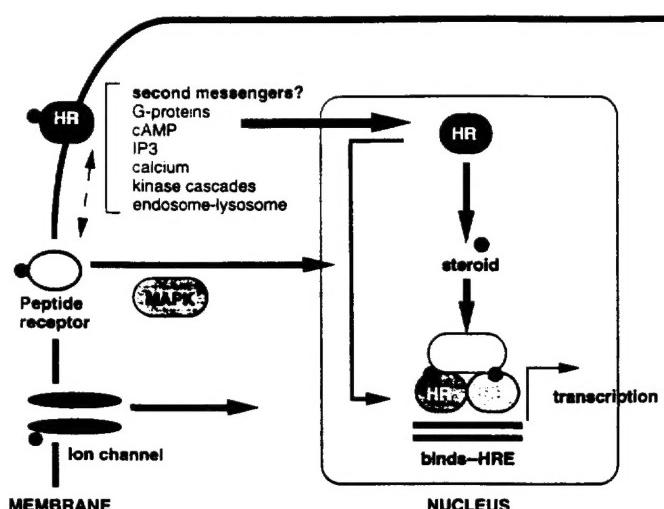


Fig. 1. Postulated mechanism of action of steroid hormones (black circles) in target cells with steroid hormone receptor (HR). In most current models, steroid binding to HR is believed to promote alterations in receptor conformation favoring enhanced association with coactivator proteins and with specific hormone-responsive elements (HRE) in the nucleus, leading, in turn, to initiation of selective gene transcription. However, the latter model fails to account for numerous, rapid cell responses to steroid treatment (see Table 1 and text). These deficiencies in the genomic model of hormone action require integration with the latter observations. In the model shown here, steroids may also bind to a membrane HR, with potential for promotion of hormonal responses via a complementary pathway that may cross-communicate or interact directly with the genomic mechanism. As noted in the text, membrane HR may be known molecules (kinases, ion channels, other receptors) with previously unrecognized binding sites for steroid, new isoforms of HR in membranes, "classic" forms of HR complexed with other membrane-associated proteins, truly novel membrane proteins, or a combination of these. Available evidence indicates that liganded membrane HR may affect one or more of several pathways, including modulation of ion channels, leading to enhanced flux of ions, notably Ca^{++} ; interaction with peptide membrane receptors; and activation of G-proteins, nucleotide cyclases, and MAPK, with resultant increases in their catalytic products (see Table 1). These membrane interactions may promote phosphorylation of HR itself via steroid-induced or ligand-independent pathways. The intricate array of physiologic responses of cells to steroid hormones may occur as a consequence of a synergistic feed-forward circuit in which steroids activate cell membrane signaling pathways that act, in turn, to enhance the transcriptional activity of HR (Table 1). Active reconsideration of the unqualified genomic model of nuclear receptor action is ongoing, and the probable importance of alternate signaling pathways elicited by surface recognition is now increasingly evident.

natively, it could serve to shuttle ligand to the nuclear-localized fraction of receptor. As has frequently been noted from these laboratories (cf. ref. 105), the cellular mechanisms governing the further transport and targeting of signaling molecules are powerful avenues of current investigation.

Many issues remain to be resolved for fuller understanding of the biologic actions of steroid hormones. Foremost among these is the structural characterization of membrane

steroid hormone receptors. It is now abundantly clear that the nuclear receptor-mediated mechanism as the sole means by which steroid hormones act is incomplete (2,3,5,7,15, 107). It is likewise unmistakable that membrane effects of steroid hormones represent an established phenomenon that is by no means to be construed as alternative to the genomic pathway, and that demands continued investigation. Indeed, the chain of membrane-initiated events is helping to account for the relatively prolonged, apparent silence between the capture of the hormone at the surface of its preferential target and the eventual outcome in augmented genomic activities. In challenging the dogma that steroid hormones act exclusively via intracellular receptors, the membrane receptor experiments reviewed here provide a persuasive paradigm for a potentially new class of drugs for human therapy. The clinical use of steroid hormone agonists and antagonists has substantially changed the course of many hormone-related diseases, but side effects of many agents currently in use are also significant. In-depth analysis of the relative contributions of nuclear and membrane-initiated activities in steroid receptor biology may lead to the development of pharmaceutical agents that exert differential activities in the two pathways, thus favoring more selective drug delivery and promoting the emergence of novel approaches for treatment of many cell metabolic and proliferative defects.

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